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Size-exclusion chromatographic determination of β-glucan with postcolumn reaction detection

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ABSTRACT

Using the specific interaction of β-glucan with calcofluor dye as a detection method for size-exclusion chromatographic analysis, very simple direct extractions can be used for sample preparation. Either fluorescence or UV detection is employed. The calibration graphs are linear between 200 and 20 mg/l, although much more sensitive analyses are possible. The response remained independent of relative molecular mass in the range studied (1.7 10^6–1.85 10% Results for barley and malt show good correlation with those obtained by flow-injection analysis using calcofluor.

INTRODUCTION

The major soluble component in barley and oat cell walls is (l-+3),(1 →4)-β-D-glucan. Most of the interest in oat β-glucan has been attributed to its possible role in lowering serum cholesterol levels [1]. It is also a good source of soluble fibre and in recent studies purified soluble fibre extract (oat gum) has been demonstrated to reduce postprandial glucose and insulin increases [2]. Jenkins et *al.* [3] have reported that a positive correlation exists between viscosity and the efficiency of soluble fibres in this reduction, hence oat gum with higher relative molecular mass components should be the preferred product.

On the other hand, in barley β-glucan is considered to be responsible for several problems in the brewing industry, including delayed filtration of wort and beer and gel precipitations in lagering. These problems are related not only to β-glucan content, but also to its relative molecular mass (M_r), especially in the concentration of the β-glucan fraction which is soluble in perchloric acid [4].

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There are two widely used methods for β-glucan determination. One is based on the utilization of specific enzymes and the determination of released oligosaccharide or glucose [5]. The other is based on the specific interaction of β-glucan with calcofluor dye [6–8]. This interaction results in an increase in fluorescence intensity and also in a bathochromatic shift. The increase in fluorescence has been widely employed in flow-injection analysis (FIA) of β-glucan in beer and wort [9–12], and also in the determination of β-glucan fractions isolated by size-exclusion chromatography (SEC), both off-line [13–15] and recently for on-line post-column detection [16]. SEC of β-glucan has been studied by Vårum et *al.* [17], who showed that its structure is more rigid than that of dextran or pollulan, and hence their utilization as standards would result in servere overestimation of the relative molecular mass of β-glucan. Similar results were obtained also by Wood et al. [16].

The injection of highly viscous samples easily results in deformation of peak shapes and thus in erroneous results. This effect is most serious in SEC as dilution is less than in other chromatographic modes [18]. β-Glucan is one example of samples that have high viscosity and thus may easily result in erroneous results because of deformed peaks. We

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encountered this effect in our initial experiments with a multi-angle laser light-scattering detector, where in some instances no change in molecular size could be seen with increasing elution volume. This effect could also be seen by the much improved peak shape on decreasing the sample concentration from 2000 to 200 mg/l [19]. This level is near the detection limit of the refractive index detector. β-Glucan samples are also easily contaminated by other polysaccharides, especially if more alkaline extraction conditions are used. Hence universal detection with a refractive index detector is by no means adequate without extensive cleaning of samples.

We have previously employed 50 m*M* sodium hydroxide solution as an SEC eluent for starch samples [20] and showed that the chromatographic conditions used give a linear calibration graph with pullulan standards in M_r range 853 000-5800 [21] and with a separation potential for much larger molecules. The employment of this elution system in connection with specific detection using calcofluor dye led us to a system for the determination of β-glucans in which aqueous buffers of any pH may be used for sample extraction followed by direct injection of the sample into the HPLC system. As we utilize the bathochromatic shift that occurs on interaction of β-glucan with calcofluor, the method has a much lower background signal than the on-line postcolumn fluorimetric method utilizing only the increase in the fluorescence signal caused by the interaction of calcofluor with β-glucan. Hence our method works in the concentration range 200–20 mg/l and UV detection can also be used in addition to fluorimetric detection.

The good sensitivity also results in a sample/extractant ratio of 80–300, which in connection with the minimum sample treatment required results in high recoveries and reproducibility. Some results obtained with this method have been presented previously [22].

EXPERIMENTAL

Chemicals

Oat β-glucan standard compounds were isolated and their M_r were determined as $1.50 \cdot 10^6$ and $4.6 \cdot 10^5$ by measurement with a Malls detector (Dawn, Santa Barbara. CA. USA). Barley β-glucan with M_r = *1.85* 10^5 was obtained from Biocon (Sydney, Australia). Wheat and rye arabinoxylans were obtained from Megazyme (Cork, Ireland), birch xylan from Roth (Karlsruhe, Germany) and calcofluor (Fluorescent Brightener 28) from Aldrich Chemie (Steinheim, Germany). The water used was distilled water passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All the other chemicals were of analytical-reagent grade. Milled barley and malt samples were obtained from the Biotechnical Laboratory of the Technical Research Centre of Finland and the oat bran samples were from our laboratory.

Equipment

The chromatographic system consisted of an M-590 pump, an M-715 automatic injector and μHydrogel 250 and 2000 columns in series in a column oven maintained at 65°C. Calcofluor solution (30 mg/l in 50 m*M* NaOH) was fed into the eluent by a reagent delivery module (RDM) at a flow-rate of 0.3 ml/min. For detection an M-490 fluorescence detector with $\lambda_{ex.}$ = 415 nm and $\lambda_{em.}$ = 445 nm at a sensitivity setting of 10 × was employed, together with an M-991 diode-array detector monitoring at 415 nm. The instrument was controlled and data were handled by an M-820 Maxima workstation (all equipment from Millipore-Waters).

The eluent was 50 m*M* aqueous NaOH at a flow-rate of 0.5 ml/min and was continuously purged with helium.

Sample preparation

A 30-mg sample of milled barley or malt was mixed either with 20 ml of 50 m*M* NaOH solution (procedure A) or 20 ml of 50 m*M* sodium phosphate buffer (pH 11) (procedure B). A 300-mg sample of milled barley or malt was mixed with 20 ml of 1 A4 NaOH (procedure C), and a 100-mg sample was mixed with 18 ml of 50 m*M* perchloric acid (procedure D). All the samples were stirred overnight in a magnetic stirrer. The only sample preparation before injection of 50-μl samples was dilution 1:10 with water of the samples prepared according to procedure C. Oat bran samples were prepared in a similar manner.

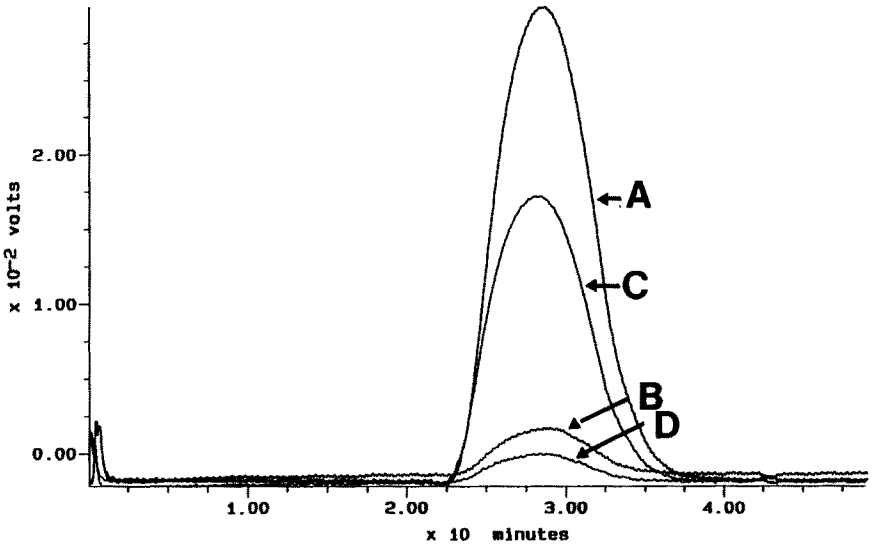

Fig. 1. SEC analysis of β-glucan standards containing 180 and 18 mg/l of β-glucan using (A and B) fluorescence detection and (C and D) UV detection. For other conditions, see text.

RESULTS AND DISCUSSION

The calibration graph based on the measurement of the area of the β-glucan peak was linear between β-glucan concentrations of 200 and 20 mg/l for both fluorescence and UV detection (Fig. 1). Both wavelengths chosen were optimum regarding the signal-to-noise ratio, which is mostly determined by the evenness of the flow of the calcofluor solution. At higher concentrations the calibration graph started to flatten because of too high a ratio of β-glucan to calcofluor. Attempts to increase the linear range by using more concentrated calcofluor solution resulted in a problem with the baseline. With lower concentrations the noise of the baseline started to affect the linearity, although as we are utilizing the bathochromatic shift the background noise is less problematic than when working at lower wavelengths. The background signal may be reduced by diluting the calcofluor reagent and using a higher sensitivity setting of the fluorescence detector. Thus the the detection limit may be reduced to 0.2 mg/l but the linearity of the calibration graph is inferior to that obtained in the concentration range 200–20 mg/l.

The relative standard deviations for the retention time of standards in a run lasting 70 h were 0.36% and 0.17% (n = 8), although the first value was obtained for a broader peak, which may be the reason for the higher standard deviation.

The response for β-glucans in this relative molecular mass range remained independent of the latter. First the hydrolysed β-glucan sample having an estimated M_r of 30 000 showed a peak with a steeply dropping end, which indicates reduced interaction with calcofluor with small β-glucan molecules, as mentioned by Foldager and Jørgensen [13] and Manzanares *et al.* [15]. The addition of 0.1 M NaCl to increase the ionic strength of the calcofluor reagent, as suggested by Manzanares *et al.*, did not have any noticeable effect on the peak tailing, but this may be because our samples still had too high relative molecular masses to be affected by this phenomenon. Manzaranes *et al.* [15] noticed this phenomenon already for β-glucans having M_r < 200 000, but they calibrated their column with dextran so their relative molecular mass was erroneously high.

The relative standard deviation for eight standards run during a 70-h analysis series was 2.2%. A comparison between the β-glucan concentration calculated on the basis of the peak area in chromatograms from barley and malt samples prepared according to procedure B and with ordinary FIA using calcofluor is presented in Table I. As can be

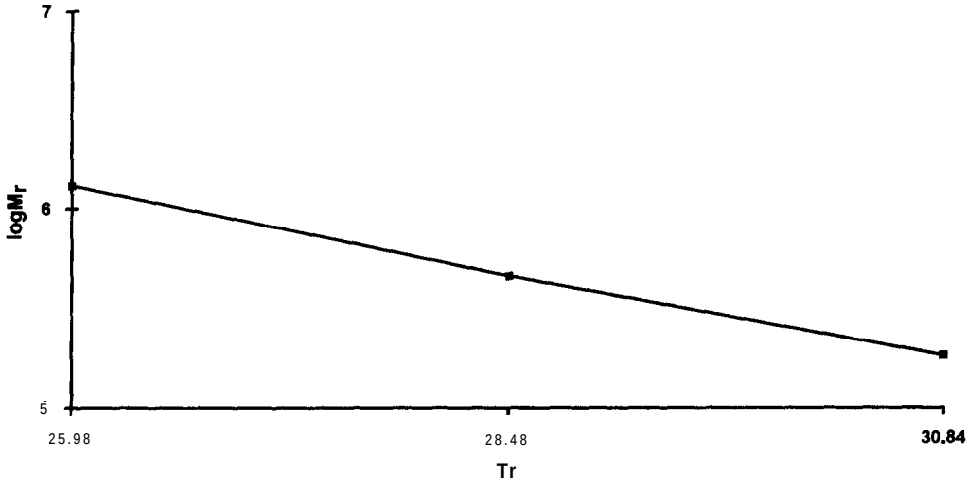

Fig. 2. SEC calibration plot. Tr in minutes.

seen, the results are very similar. The relative standard deviation for parallel samples was 2.3% with different extraction procedures. The β-glucan concentration obtained by procedure B varied from 19 to 47% in comparison with that obtained with procedure A. With procedure C the values varied from 77 to 94% and for procedure D, which measures more the concentration of β-glucan that causes problems in beer manufacture, the values varied from 32 to 53%. As can be seen in Fig. 4, the relative molecular mass of these perchloric acid-soluble β-glucans is smaller than that in the fraction soluble in alkali. Figs. 3 and 4 also reveals that the relative molecular mass of β-glucan is not reduced during

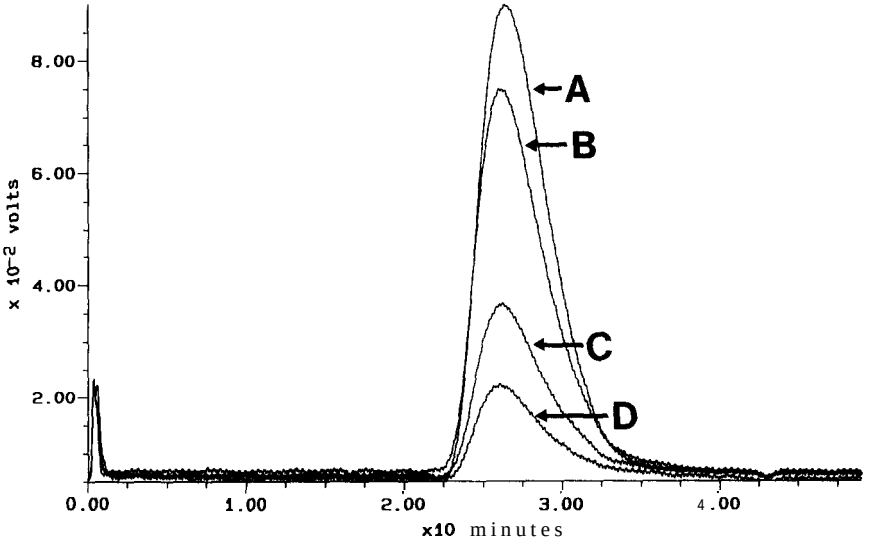

Fig. 3. Chromatogram of (A) Kymppi barley and (B-D) malts made from it during different stages of malting. Fluorescence detection. For other conditions, see text.

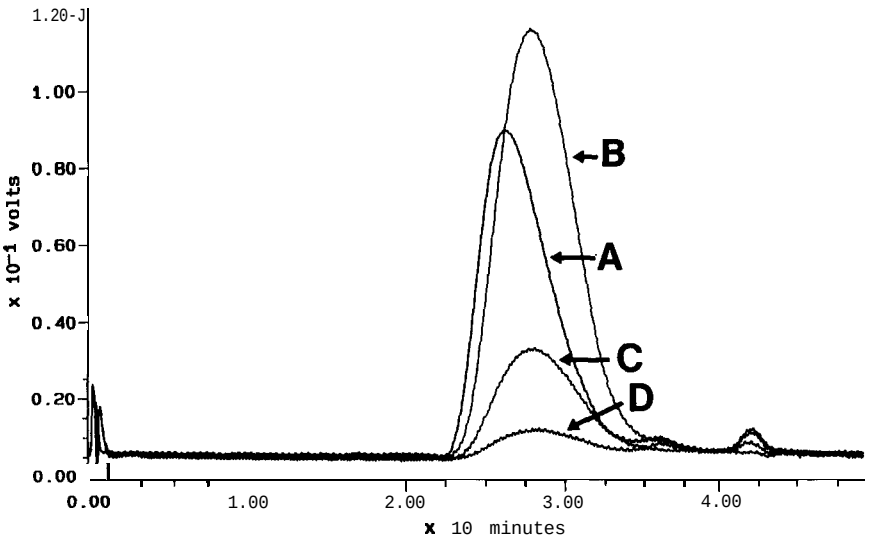

Fig. 4. Chromatogram of Kymppi barley with (A) alkaline extraction and (B) perchloric acid extraction. Chromatograms C and D are from perchloric acid extractions of malts made from Kymppi barley. Sample amounts for perchloric acid extractions were three times higher than for alkaline extraction

malting, although the amount is reduced. The hydrolysis results in such small molecules that they are no longer stained by calcofluor.

For the oat bran, procedure A again gave the highest values, although the values from procedures B and C ranged from 90 to 95%, which may reflect the easier accessibility to β-glucan in oat bran than in barley. The values for bran ranged from 55 to 47 g/kg.

TABLE I

DETERMINATION OF β-GLUCAN CONCENTRATION BY THE CHROMATOGRAPHIC METHOD DESCRIBED AND BY THE FIA METHOD WITH CALCOFLUOR

Sample	β-Glucan concentration (g/kg)	
	SEC	FIA
Barley		
Kymppi	*48*	*48*
HJA	45	45
Mut1460	24	24
Mut737	31	35
Minerva	48	51
Prisma	34	32
Malt		
SH 16	33	38
SH 17	17	17
SH 18	8	9

Wood [8] has reported that calcofluor interacts with some other polysaccharides such as xylans. Some xylans and arabinoxylans were studied using the present chromatographic system but none of them showed any response in the relative molecular mass range considered.

The use of strongly alkaline solutions for extraction may also dissolve starch, so its possible effect was also studied. A 40-fold excess of starch in the β-glucan standard did not have any effect on the standard peak.

The method presented gives the possibility of studying the concentration and relative molecular mass of β-glucan in barley, malt and oat with minimum sample preparation and with good accuracy.

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